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(54) Method of extracting tumor necrosis factor-like substance.

(57) Disclosed herein is a method of extracting a large amount of pure tumor necrosis factor-like substance from fibroblast, epithelial or lymphoblast-like cells killed by an overpopulation in a culture medium or destroyed in a culture medium by an artificial treatment such as a quick freezing and quick thawing. The pure tumor necrosis factorlike substance has the cytotoxicity or cytostaticity to tumor cells.

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The objects of this invention are to extract TNS simply and a lot in quantity. The method is the following:

(1) Maintain in culture a sort of cell of a fibroblast, epithelial or lymphoblast-like cell.

(2) Kill the cultured cell, naturally or artificially.

(3) Extract TNS from the killed cell.

The inventor of this invention produced in success a large amount of pure TNS from cultured L-929 fibroblasts, and found that TNS was present in supernatant obtained from centrifugation of fibroblast-like cells after step (1) and (2). The following is described in details; as step (a), a macrophage cell line of CAMU-3-R or a fibroblast cell line of L-929 are homogeneously killed in culture medium. As step (b), the medium in step (a) is mixed with a tumor cell line of Meth A, and this cells are incubated for 48 hours. As step (c), the culture medium containing the killed fibroblast cell line is mixed and incubated with the tumor cell lines of a Meth A for 48 hours. As step (d), the killing effect of the fluid in step (c) which contains a macrophage cell line is compared with that of fibroblast cell lines. This percent index means the cytotoxicity or cytostaticity to tumor cells. The percent indices of the macrophage, fibroblast and artificially destroyed cell lines are 8.8%, 83.7% and 96.9%, respectively. The fluid of a higher percentage was not cytotoxic to normal cells. This result was confirmed by a production of a large amount of TNS in cells in a fibroblast, an epithelial and a lymphoblast-like cell. The TNS extraction from fibroblast, epithelial and lymphoblast-like cells is significant.

Culture of fibroblast, epithelial or lymphoblast-like cells obtained in vivo is more simple than of cultured macrophages. These cells are more productive without consuming time than macrophages. TNS from a definite cell is more pure than that from animals. Next, this invention confirmes a biological role of the TNS as a curable drug for cancer.

Table 1

Culture Medium with Supernatant in Concentration Ratio of	48 Hour-CTA of a Meth A Sarcoma	
	Number of Living Cells ($x10^4$)	CAT (%)
1 to 0	68	0
0.9 to 0.1	32.7	44.6
0.8 to 0.2	14.0	79.4
0.7 to 0.3	8.2	87.9
0.6 to 0.4	6.7	90.1
0.5 to 0.5	5.6	91.8

[III] Biological characteristics of TNS

(A) The supernatant [I]-(5) was heated at 56°C for 30 minutes, at 70°C for 60 minutes, and at 85°C for 15 minutes, respectively. See Table 2. The negative relationship between CTA and heating was noted.

Table 2

Heating Conditions of Supernatant	48 Hour-CTA of a Meth A Sarcoma	
	Number of Living Cells ($x10^4$)	CTA (%)
at 56°C for 30 Minutes	14.2	76.3
at 70°C for 60 Minutes	49.5	17.5
at 85°C for 15 Minutes	43.0	28.3
Culture Medium without Supernatant, without a Heating Treatment	60	0

This result indicates that TNS extracted from CAMU-3-A7 is likely to be a glycoprotein, because CTA decline with

Table 3B
(continued)

	CTA According to Concentrations of CAMU-3-A7 in Cell Suspension	
	Number of Living Cells ($x10^4$)	CTA (%)
Concentration of CAMU-3-A7 Cell Suspension $4x10^6$/ml	1.3	98.4

The explanation about [II] is as follows: [II-1] Epithelial like-cells of a CAMU-3-A7 cells cultured in a petri dish. [II-2] Detach cells from the petri dish face using tripsin or EDTA. [II-3] mix the culture cells in culture medium such as MEM, RPMI1640 or KN7 contaning 5% newborne calf serum and adjust the cell concentration of $3x10^6$/ml in suspension. [II-4] Put the cell suspension into a cryotube and freeze quickly in liquid nitrogen and keep it for 5 minutes. [II-5] Thaw it in 37°C hot water for 10 minutes. [II-6] Repeat [II-4] and [II-5] three times. [II-7] Centrifuge the solution [II-6] at 2000 r.p.m. for 10 minutes, and separate the supernatant, which may contain TNS.

In comparison of TNS [I] and TNS [II], each CTA was identical and the same CTA was obtained from the supernatant of the lymphoblast-like cell (RLô-1) or the fibroblast-like cell (L929) instead of CAMU-3-A7. See Table 4.

Table 4

	Comparison between CTA of TNS [I] and TNS [II]	
	Number of Living Cells ($x10^4$)	CTA (%)
Culture Medium without Supernatant	78	0
Culture Medium with Supernatant Contained Killed CAMU-3-A7 Cells	2.4	96.9

and destroyed by a quick freezing and thawing. A special membrane which can be filtered less than a molecular weight of $1x10^4$, $3x10^4$ or $1x10^5$ respectively was used in order to confirm the molecular weight of TNS in the supernatant. Table 5 shows the results of this experiment.

Table 5

	Comparison of CTA on Tumor Cells of a Meth A Sarcoma Cultured for 48 Hours.	
	Number of Living Cells ($x10^4$)	CTA (%)
Culture Medium without Supernatant	77	0
Less Than Molecular Weight of $1x10^4$	66	14.3
Less Than Molecular Weight of $3x10^4$	67	13.0
Less Than Molecular Weight of $1x10^5$	77.5	0
More Than Molecular Weight of $1x10^5$	4.0	94.8

As is evident from Table 5, the molecular weight of TNS is expected to be more than $1x10^5$.

The following experiment was tried to confirm CTA on tumor cells of a Meth A sarcoma cultured for 48 hours in a culture medium mixed with a supernatant produced from several kinds of cells. The results of this experiment are shown in Tables 6 to 8.

Table 7
(Continued)

	Number of Living Cells ($x10^4$)	CTA (%)
Culture Medium with Supernatant Produced from a SV-T2 Adjusted to $3x10^6$/ml and Destroyed by a Quick Freezing and Thawing	64.0	13.5

Table 8

	Number of Living Cells ($x10^4$)	CTA (%)
Culture Medium without Supernatant	83.5	0
Culture Medium witn Supernatant Produced from a L929 Adjusted to $3x10^6$/ml and Destroyed by a Quick Freezing and Thawing	2.0	97.6
Culture Medium with Supernatant Produced from a Balb/3T3 Adjusted to $3x10^6$/ml and Destroyed by a Quick Freezing and Thawing	1.8	97.8
Culture Medium with Supernatant Produced from a Normal Mouse Fetus Adjusted to $3x10^6$/ml and Destroyed by a Quick Freezing and Thawing	20.7	75.2
Culture Medium with Supernatant Produced from a YAC-1 Adjusted to $3x10^6$/ml and Destroyed by a Quick Freezing and Thawing	82.5	1.2

Table 9
(Continued)

	Comparison of CTA on the Tumor Cells of a Meth A Sarcoma Cultured for 48 Hours, in case of Using a CAMU-3-R	
	Number of Living Cells ($x10^4$)	CTA (%)
Culture Medium with Supernatant Produced from a CAMU-3-R Adjusted to $5x10^6$/ml	61.8	0
Culture Medium with Supernatant Produced from a CAMU-3-R Adjusted to $6x10^6$/ml	70.5	0
Culture Medium with Supernatant Produced from a CAMU-3-R Adjusted to $7x10^6$/ml	59.5	0
Culture Medium with Supernatant Produced from a CAMU-3-R Adjusted to $8x10^6$/ml	60.5	0

Table 10

	Comparison of CTA on the Tumor Cells of a Meth A Sarcoma Cultured for 48 Hours, in case of Using a CAMU-31-R	
	Number of Living Cells ($x10^4$)	CTA (%)
Culture Medium without Supernatant	56.5	0
Culture Medium with Supernatant Produced from a CAMU-31-R Adjusted to $4x10^6$/ml	46.5	17.7
Culture Medium with Supernatant Produced from a CAMU-31-R Adjusted to $5x10^6$/ml	49.8	11.9

Table 11

	Comparison of CTA on Normal Cells					
	Normal Cells Separated from a Spleen of Balb/c Mouse : Lymphatic-Like Cell ($1x10^6$/ml)		Normal Cells Separated from a Fetus of Balb/c Mouse : Lymphatic-Like Cell ($5x10^5$/ml)		Normal Cells Separated from a Fetus of Balb/c Mouse : Fibroblast-Like Cell ($5x10^5$/ml)	
	Number of Living Cells ($x10^4$)	CTA (%)	Number of Living Cells ($x10^4$)	CTA (%)	Number of Living Cells ($x10^4$)	CTA (%)
Culture Medium without Supernatant	24.2	0	77.5	0	35.5	0
Culture Medium with Supernatant Produced from a CAMU-3-A7 Adjusted to $3x10^6$/ml and Cultured for 96 Hours	52	0	82	0	41.3	0
Culture Medium with Supernatant Produced from a L929 Adjusted to $3x10^6$/ml and Cultured for 96 Hours	55	0	-	-	-	-

As is evident from Table 11, supernatants shown in Table 11 do not have CTA on normal cells.

The following results are shown in the above mentioned experiments.

1. Factor having a high percentage of CTA on tumor cells is presents in supernatant produced from killed or destroyed epithelial-like, fibroblast-like or lymphoblast-like cells.

2. The factor is effective on tumor cells under the circumstances of up to the heating temperature of 56°C for 30 minutes. It also loses CTA on tumor cells under the circumstances of more than the heating temperature of 70°C

CLAIMS

1. A method of extracting a tumor necrosis factor-like substance from cells which comprises incubating at least one type of cell selected from fibroblast, epithelial and lymphoblast cells in a culture medium, killing said cells naturally in said culture medium, and extracting a tumor necrosis factor-like substance from said culture medium contained in said killed cells.

2. A method of extracting a tumor necrosis factor-like substance from cells which comprises incubating at least one type of cell selected from fibroblast, epithelial and lymphoblast cells in a culture medium, destroying said cells in said culture medium by rapid freezing and thawing, and extracting a tumor necrosis factor-like substance from said culture medium contained in said destroyed cells.

3. A method according to claim 2 in which the rapid freezing and thawing comprises supersonic treatment.